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Recent Advances in Therapeutic Delivery Systems of Bacteriophage and Bacteriophage-Encoded Endolysins

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Abstract

Antibiotics have been the cornerstone of clinical management of bacterial infection since their discovery in the early 20th century. However, their widespread and often indiscriminate use has now led to reports of multidrug resistance becoming globally commonplace. Bacteriophage therapy has undergone a recent revival in battle against pathogenic bacteria, as the self-replicating and co-evolutionary features of these predatory virions offer several advantages over conventional therapeutic agents. In particular, the use of targeted bacteriophage therapy from specialised delivery platforms has shown particular promise owing to the control of delivery location, administration conditions, and dosage of the therapeutic cargo. This review presents an overview of the recent formulations and applications of such delivery vehicles as an innovative and elegant tool for bacterial control.

Key Words: Bacteriophage, Endolysin, Encapsulation, Immobilisation, Stimuli Responsive, Controlled Release, Complexation, Delivery System

Introduction

Following the discovery of penicillin in 1928 by Alexander Fleming and the subsequent development of modern antibiotics, the World has enjoyed a period of relative safety from the threat of bacterial infection. However, Fleming himself was discerningly cautious about the potential of modern antibiotics to keep this threat at bay, going on to describe the possibility of developed resistance in his 1945 Nobel lecture detailing the dangers of “underdosage” [1]. This predicted evolution of multidrug resistant bacteria, and the subsequent decline in the production of novel antibiotics has driven a resurgence of interest in the once forgotten viral therapies of the Eastern Bloc countries. Bacteriophage (phage) have been developed for therapeutic use since their discovery in the early 20th century, dominating antimicrobial treatment in the East where the antibiotic panacea failed to translate as effectively [2,3]. As ubiquitous predators of bacteria, phage exist in the biosphere alongside their host, continuously engaged in a biological game of cat and mouse, resulting in an estimated 50% reduction in the global bacterial population every 48 hours, arising from phage predation alone [4]. Phage propagate in bacteria through lytic development or lysogenic replication (Figure 1) [5]. For use as therapeutic agents, lytic phage (incapable of lysogenic infection and more potent than temperate phage) are identified by phenotypic and structural characterisation as well as genetic sequencing, in order to avoid any possible genetic transfer events, such as transduction.

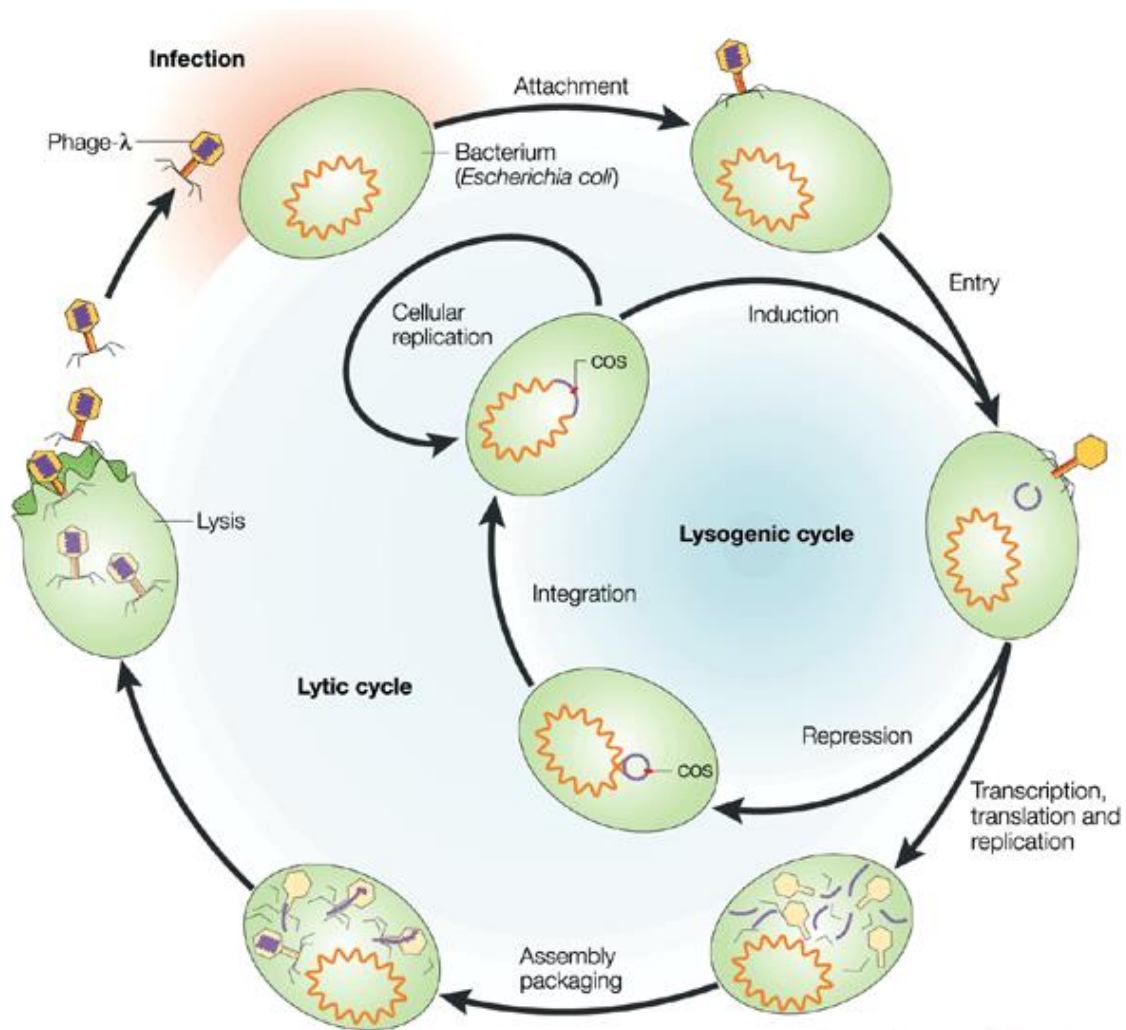


Figure 1 – Standard viral replication cycles of bacteriophage. The closure of the newly formed circular DNA and corresponding cohesive site is denoted ‘cos’. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics [5], copyright 2013.

As host specific infectious agents, phage have been investigated as a form of bacterial biocontrol, with a range of applications in the medical, agricultural and biotechnology industries [6-9]. Owing to their high natural abundance, phage are relatively easily sourced and have been successfully isolated from all ecological environments in which bacteria are also present [10]. When used in combination as a *phage cocktail*, they are able to infect and destroy a multitude of bacterial species including both Gram-positive and Gram-negative

strains. These self-replicating virions are also active against antibiotic resistant isolates such as *Pseudomonas aeruginosa* (*P. aeruginosa*), a multidrug resistant pathogen associated with a range of infectious diseases [11]. There have been a number of phage based products approved by the FDA within the agricultural sector including; ListShield™ for use on ready to eat meat, seafood and food contact surfaces, SalmoFresh for poultry, fish and fruit and vegetables, EcoShield for red meat and LISTEX™ for meat, fish and cheese [12]. However, there are currently no licenced bacteriophage or bacteriophage derived products approved for human therapeutic use in the EU or the USA.

The need for regulated clinical trials and compliance with current manufacturing guidelines is a major complication in the implementation of sustainable phage therapy. The reliability of early studies from previously inaccessible countries (such as those within the former Soviet Union), have since been called into question based on factors such as lack of blinding and co-administration with antibiotics during early experimental therapy [13]. However in response to the ever-pressing requirement to develop new and more effective antimicrobials, the development of phage based products has recently seen some promising pre-clinical results, with a number of clinical trials underway. PhagoBurn (Trial Number NCT02116010 [14]), funded by the European Union is currently undergoing phase I/II trials utilising a phage cocktail to treat burn wound infections with results expected in March/April 2017. AmpliPhi Biosciences have a phage product AB-SA01 (Trial Number NCT02757755 [15]), active against *Staphylococcus aureus* (*S.aureus*) entering into phase II trials for the treatment of both chronic rhinosinusitis and bacterial skin infection. Previous clinical trials adhering to modern protocols have also seen success in the treatment of a range of infections, including venous leg ulcers, otitis and reduction of bacterial load in the nasal carriage [16-18]. In addition to

regulatory and clinical requirements, the pharmacokinetics (PK) and pharmacodynamics (PD) of phage therapy must be considered. The administration of an antimicrobial at the site of infection must be both timely and in sufficient concentration to elicit a response, thus avoiding effects such as sub-lethal dose administration (with associated resistance development), immune response/ clearance, and diffusion limitations [19]. The physiological stability of such biological entities must be maintained during treatment, including the prevention of neutralization (by antibodies or other such compounds), and protein degradation via localised environmental conditions [20]. The trials described above all focus on topical application of phage. One of the outstanding questions regarding their suitability for treatment of a wider range of infections is their suitability for systemic delivery. To be able to apply the phage distant to the site of infection necessitates a greater understanding of the clearance of phage from the body and strategies to maintain an effective concentration long enough for phage attachment to the pathogen and subsequent infection and amplification. Both the innate and adaptive immune system have a role in reducing the level of circulating phage. This has been discussed in detail recently [6] and is outside of the scope of the current review. This is likely to be a key determinant of therapeutic efficacy, but a number of the encapsulation and delivery methods described below also help to reduce immune clearance as well as enhancing other aspects of PK/PD. Therefore, the development of suitable delivery vehicles has received considerable attention recently in order to improve and maintain the pharmacological properties of therapeutic phage products *in vivo*.

A range of techniques have been employed in recent years utilizing various technologies (encapsulation, immobilization, conjugation etc.) to successfully exploit bacteriophage for a number of applications including; sensing and detection of infection, bacterial capture and

phage-assisted delivery of therapeutic cargo [21-26]. The bacterial acquisition of specific genes via phage guided delivery, using CRISPR-Cas technology, for sensitization of previously drug resistant bacterial isolates [27,28], and the delivery of genes expressing novel antibacterial agents (SASPject; [29]) has demonstrated the versatility and extent to which phage have the potential to be utilized in modern medicine. This review will focus on the current advancements in delivery systems capable of housing phage for the specific therapeutic purpose of treating bacterial infection, with particular emphasis placed on the stabilisation and protective strategies required.

Encapsulation

The successful administration of a therapeutic agent to a target site often depends on a suitable delivery vehicle to ensure it reaches the infection site. An example of this is pulmonary delivery; an area in which phage have received considerable attention in recent years, owing to the increase in multidrug resistance amongst bacterial isolates, including those associated with pneumonia and tuberculosis. *Klebsiella pneumoniae* (*K. pneumoniae*) is a major cause of nosocomial pneumonia, especially amongst immunocompromised patients with reported mortality rates as high as 60% [30]. Whilst previously considered an extracellular pathogen, this Gram-negative bacterium has shown the ability to become internalised *in vivo* by certain cell types including lung epithelial cells [31]. Therefore, effective treatment requires intracellular access, a concept referred to as the 'Trojan horse' approach, relying on a suitable vector to transport phage across the eukaryotic cell membrane, delivering the antimicrobial cargo directly into the infected cell. The ability of bacteria to colonise intracellularly, serving as a reservoir of infection and the inability of

phage to enter myeloid cells, has previously rendered phage therapy ineffective for certain conditions. However the use of lipid-based carriers as a means of transportation across the cell membrane may offer the possibility of phage as a viable treatment option. Liposomal entrapment of the fully characterised lytic phage KPO1K2 appears to provide enhanced intracellular uptake into phagocytic cells in order to target engulfed *K. pneumoniae* present within macrophages. In the case of encapsulated phage, ~95% of the intracellular bacteria was eradicated within 24 hours compared to no significant difference in intracellular bacterial content using free phage (Figure 2) [32].

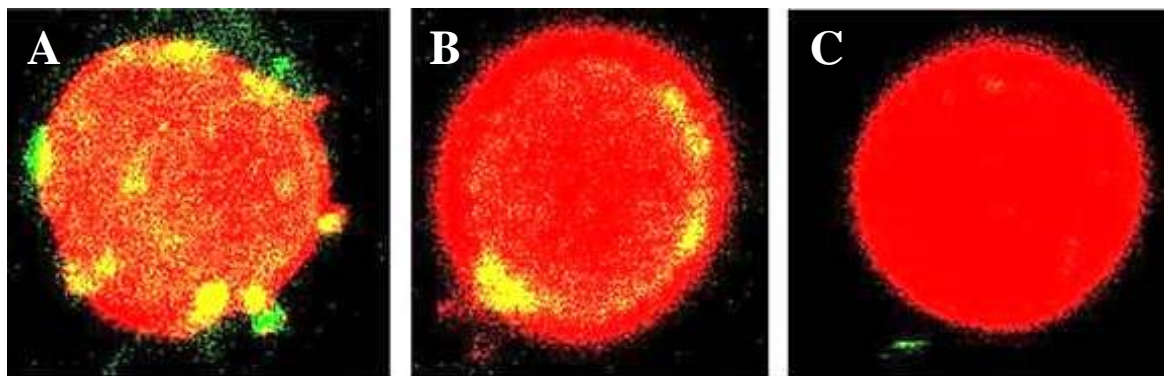


Figure 2 – Confocal micrograph showing intracellular bacterial content using Live/Dead fluorescent staining **A)** Untreated macrophages, showing intracellular bacteria in yellow **B)** Infected macrophages treated with free phage, but with intracellular bacteria still evident **C)** Liposome treated macrophages with apparent intra cellular eradication of bacteria. Figure adapted from [32].

Liposomal entrapment has also been utilized for the potential intracellular treatment of tuberculosis via successful encapsulation of the mycobacteriophage TM4 or the reporter bacteriophage λ eyfp into giant unilamellar vesicles (GUVs), by a variety of preparation techniques. Enhanced uptake into eukaryotic cells (THP-1 macrophages) was seen with

encapsulated phage compared to free phage, and no sign of mechanical damage to the liposome was observed as a result of phage encapsulation [33].

Encapsulation of phage for the purpose of treating pulmonary-associated infection has shown additional benefits in terms of neutralisation protection. Cationic liposomal entrapment appears to offer a protective barrier from which phage are able to evade anti-phage antibodies. Antibodies produced by the mammalian immune system in response to phage therapy have shown the potential to render phage inactive, a problem which appears to be dependent both on the type of phage used and the delivery route chosen [34]. Liposome encapsulated phage (active against *K. pneumoniae*) incubated alongside bacteriophage antibodies from mouse serum, experienced complete protection from any degradative effects, whereas unencapsulated phage appeared to undergo complete neutralisation, with no active phage particles remaining after 3 hours incubation *in-vitro*. [32].

Moreover, previous studies have shown that the efficacy of phage therapy in animal models is effective only after almost immediate administration post infection, whereas liposome entrapped phage appear to retain antibacterial activity against pulmonary associated infection even when treatment is delayed for up to 3 days post infection [35]. Furthermore, liposomal encapsulation of phage has demonstrated a greater degree of bio-distribution and bio-retention compared to free phage. This has been shown in *in vivo* experiments via the inoculation of mice with both free phage and phage encapsulated within phospholipid vesicles. Encapsulated phage were present at higher peak levels and concentrations were maintained longer, declining after 12 hours for encapsulated phage compared to 6 hours for free phage. Phage remained detectable for up to 4 days in blood, 6 days in the liver, lungs

and kidney, and up to 14 days in the spleen following a single intraperitoneal injection, compared to undetectable levels after 48 hours in all 4 organs when using free phage (aqueous suspension) [36]. The maintenance of maximal concentration and length of persistence of phage in the circulation observed in this study, suggests that systemic treatment may be possible using such approaches.

Nanoemulsions consisting of phage within the aqueous core of a lipid suspension (water-in-oil-in-water), have gained attention, owing to the enhanced functional and structural stability of the entropically confined phage encased within such micelles. A positive correlation has been seen between the fatty acid chain length of the lipid micelle and the emulsion stability, manifested as an overall long term stability of up to 3 months at room temperature [37]. Alongside an increase in shelf life, the use of nanoemulsions has previously shown higher infectivity rates of bacteriophage when compared to aqueous phage suspensions, possibly as a result of the elimination of electrostatic repulsions between the negatively charged phage and bacteria [38,39].

Encapsulation of phage and the resulting protective effect has been exploited in the targeted delivery of bacteriophage K (active against *S. aureus*) to the intestine via oral delivery. Ensuring the successful delivery of phage (or any biological therapeutic) through the gastrointestinal (GI) tract relies on a protective strategy to prevent inactivation by stomach acid, an issue previously encountered with other encapsulation formulations, with detrimental effects observed as a function of microsphere size (<100 μm) and possible acid diffusion into the microspheres [40]. The utilisation of a microsphere delivery system consisting of alginate microspheres co-encapsulating calcium carbonate and phage K has

shown to provide a more robust protective effect against simulated GI fluid than alginate microspheres alone. Moreover, the addition of protective additives such as maltodextrin (up to 20% w/v) to the microcapsules has shown to increase the viability of phage K after drying, thus allowing for dry form encapsulated phage preparation [41]. This was further investigated using alginate-whey protein microspheres with maltose as a protective agent against dehydration effects. The encapsulated phage K exhibited enhanced stability after drying, likely as a result of the high glass transition temperature of the disaccharide: preventing denaturation by alteration of the protein dynamics through physical confinement. In addition to any benefits offered by dry formulations in this instance (ease of storage, transport and administration), dry powders containing phage exhibited storage stability at both 4°C and 23°C with over 80% of the encapsulated phage retaining viability at the higher temperature after two weeks [42]. Similarly, *Escherichia coli* (*E. coli*) O157:H7 bacteriophage have been successfully encapsulated in chitosan-alginate microspheres, demonstrating stability and sustained release under simulated gastric conditions at pH 2 and 2.5. This has potential applications in the treatment of gastroenteritis infections such as haemorrhagic colitis [43].

Immobilisation

The surface anchoring of biological entities with retention of their original function can be problematic owing to conformational changes in tertiary protein structure (denaturation) and changes in water content caused by the binding of proteins and protein ensembles to what are often rigid supports. However, the therapeutic use of phage may require immobilisation on abiotic surfaces (such as medical devices including stents, tracheal tubes, catheters etc.) since such devices are particularly prone to bacterial biofilm formation: the ability of bacteria

to colonise surfaces, forming dense and often impenetrable biofilms. A recent method targeted at successful phage immobilisation, has utilized plasma-associated activation of polytetrafluoroethylene (PTFE) and ultra-high molecular weight polyethylene (PE). Low temperature plasma treatment of such polymers created reactive acid groups which were used as anchor points for phage attachment. Such immobilised phage retained activity against both *E.coli* and *S.aureus* despite confinement at the surface. Figure 3 shows the steps involved in amide formation and subsequent phage attachment. Furthermore, the surface-bound phage retained bactericidal activity for several months under specific conditions (high humidity/ aqueous environment) [44]. This technology demonstrates the potential of phage-coated surfaces as potent antimicrobial interfaces capable of targeting important human pathogens, with the potential to prevent biofilm formation.

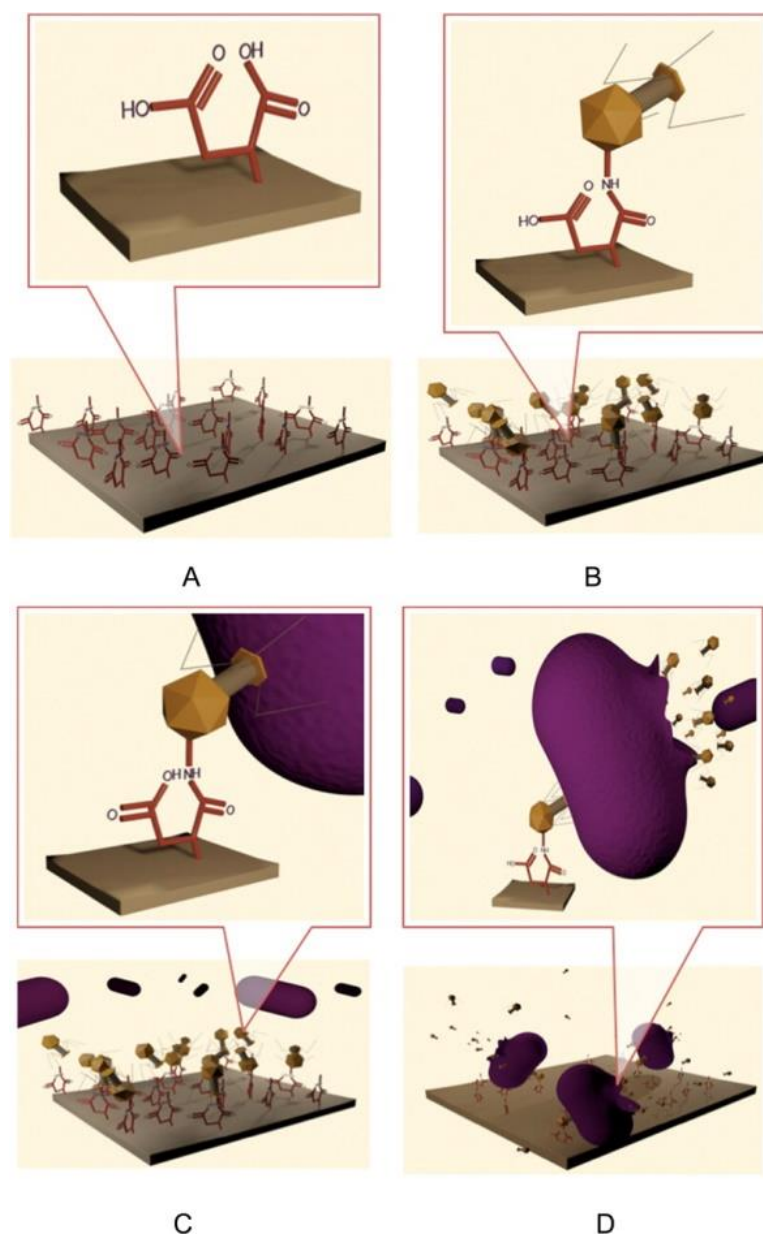


Figure 3 –Steps involved in phage anchoring for the prevention of bacterial adhesion and biofilm formation. **A)** Plasma mediated formation of surface acid groups **B)** Phage attachment via amide bond formation **C)** Phage capture of bacterial cells **D)** Bacterial lysis post phage infection. Reprinted with permission from [44]. Copyright 2013 American Chemical Society.

There are additional examples in the literature of successful phage binding to surfaces for purposes other than therapeutic treatment such as biosensors, bacterial capture platforms and antimicrobial coatings for food packaging [45-47]. Optimizing the parameters for

successful phage immobilisation in terms of surface density, orientation, infectivity and stability is paramount in the development of any phage-based surface platform. Once established, this technology has the potential to be utilized for a range of applications, including antibacterial coatings and surfaces for therapeutic use.

Polymeric Formulations

Inactivation of phage as a result of environmental factors such as temperature, pH and UV is a major hurdle in the development of phage therapy, affecting both delivery route and stability. Phage-polymer ensembles have been shown to offer beneficial effects in terms of protein stability with respect to external conditions, offering the possibility of efficient storage and therapeutic use of phage for the treatment of infection. An example of this is the use of the naturally occurring polymer, poly- γ -glutamic acid (γ -PGA). Whilst the mechanism behind the protective effect is not yet fully understood, even in very low concentration $\sim 1\%$, this biodegradable polymer has shown to effectively protect two different *E.coli* phage, MS2 and T2 (from the Leviviridae and Myoviridae families respectively), against temperatures of up to 60°C , possibly as a result of physical protection of the viral particles. Protection against UV exposure was also seen with γ -PGA formulated phage, again possibly as a result of physical protection or refraction of the radiation. In the case of the T2 phage, a significant increase in survival rate was seen at a range of pH values via polymer stabilisation [48]. This is likely due to the pH sensitive nature of the polymer itself, which undergoes a conformational change at low pH where it exists in an alpha-helical conformation, potentially encasing the phage and protecting it from the acidic environment. γ -PGA exists in a linear random-coil conformation at neutral pH, hence the protective effects at higher pH are still poorly

understood. However, further research may provide enhanced techniques for the protection of phage, with implications for both delivery and storage conditions.

Naturally occurring polymers such as proteins have shown potential to stabilise vaccine formulations, a concept which would have significant implications for the transportation and successful administration of human vaccines, especially in the developing world. Long term stability in elevated temperatures would drastically improve the often unreliable and expensive 'cold chain' transportation network required to distribute global immunisation treatment [49]. Building on this concept, recent efforts have taken the same approach using silk proteins to stabilise phage against high temperatures, showing greater efficacy in protective effects when compared to a non-silk protein (bovine serum albumin). Results obtained showed 100% loss of viable phage in the absence of protein after one day incubation at 37°C, whereas the addition of various silk proteins (honeybee, hornet and silkworm), resulted in a maximum loss of 6% viable phage (Figure 4A). Indeed, the use of honeybee silk significantly enhanced phage survival even at 50°C for up to 8 weeks (Figure 4B) [50]. A suggested reason for this is the increase in favourable protein-protein interactions, in preference of water-protein interactions within the hydration shell surrounding the phage.

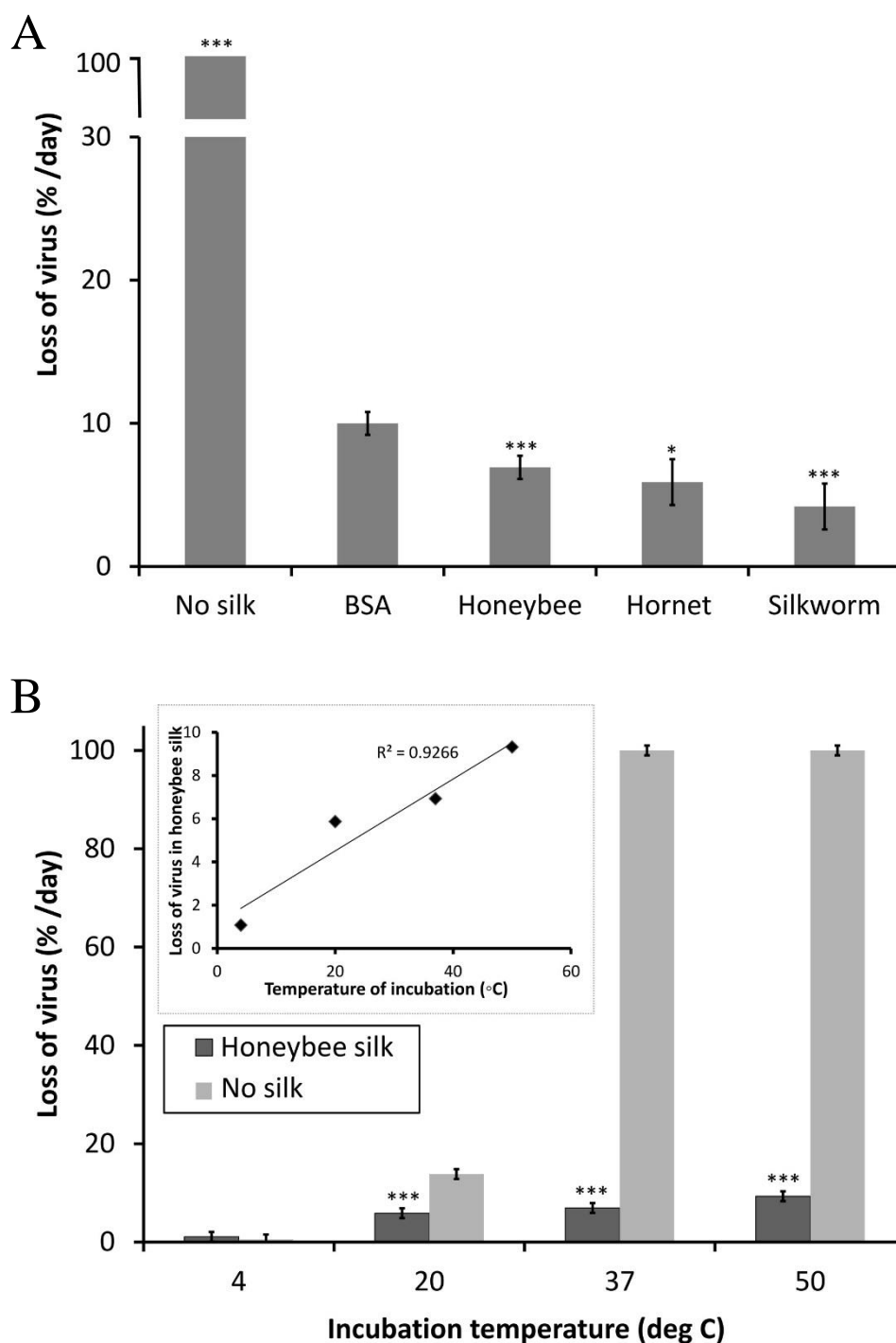


Figure 4 – Loss of viral infectivity as a function of protein addition. **A)** After incubation at 37°C with and without different proteins **B)** After incubation at various temperatures alongside honeybee silk. Inset highlights the correlation between viral viability and incubation temperature. Adapted with permission from [50]. Copyright 2014 American Chemical Society.

Aerosol Formulations

Nebulization of drug formulations is a common methodology for the delivery of pharmaceuticals and biopharmaceuticals for inhalation directly to the lung. Patients suffering from cystic fibrosis (CF) are of particular concern, owing to their high susceptibility to infection. The administration of aerosolized phage via nebulization has previously been employed in a mouse model to evaluate the efficacy of phage therapy against a group of opportunistic pathogens: *Burkholderia cepacia* complex (BCC) organisms, capable of infecting immunocompromised patients. The results obtained showed a significant reduction in bacterial load within the lungs of immunocompromised and BCC infected mice (in some cases comparable to conventional antibiotics), using a range of different phage with varying multiplicities of infection (MOI) [51]. Further investigations into the use of nebulization as a delivery system for phage treatment has focused on *P. aeruginosa*, another common pathogen associated with CF, capable of causing respiratory failure in up to 95% of patients [52]. In this case, it was determined that the amount of phage delivered to the infection site which, for an infection such as this, concerns the lower respiratory tract, the choice of nebulizer also plays a significant part. It was concluded that a jet nebulizer is most effective, as 12% of the phage contained within particles and placed within the nebulizer, were small enough ($< 4.7 \mu\text{m}$) to be inhaled into the lower lung whilst retaining viability [53].

Another important consideration in the development of any therapeutic system is the potential for scale-up operations. In order to establish a viable delivery system, one must take into consideration a number of different factors including cost, feasibility of application, convenience and downstream processing steps. Whilst nebulization has shown promising results in terms of successful delivery, there are some concerns surrounding the expense and

the complexity in the procedures associated with their use outside of the clinic (i.e. in a patient's home). As a potential alternative, recent efforts have focused on dry powder inhalers (DPIs), as a means of delivering phage in order to combat respiratory infection. Compared to nebulizers, DPIs are small, cheap, easy to operate and have been shown to successfully deliver a wide range of pharmaceuticals [54]. Aerosolized powders containing bacteriophages KS4-M and ϕ KZ, targeting both BCC and *P. aeruginosa* respectively, demonstrated successful stabilisation of the lyophilized phage alongside retention of viability utilising a 60: 40 w/w matrix of lactose/lactoferrin [55].

In addition to freeze dried formulations for use in DPIs, spray drying has been investigated for pulmonary delivery of phage as it is a less energy intensive process and requires fewer downstream processing steps. Using atomization as a potentially scalable process, two morphologically different phage, *Pseudomonas* phage LUZ19 and *Staphylococcus* phage Romulus, were successfully formulated into respirable powders in the presence of various excipients in order to protect the phage. Of those investigated, trehalose was found to be most effective in terms of protective effects, demonstrating increased stability at low temperature (4°C). However, further investigation into thermal stability showed a pronounced effect on phage viability at higher temperature (25°C) and high humidity. This is likely as a result of crystallization of the trehalose-phage amorphous matrix, thus highlighting the importance of suitable storage conditions of powder formulations containing phage particles [56]. In addition, the formation of phage-containing particles with the correct size parameters suitable for pulmonary delivery (between 1 and 5 μ m) was found to be dependent on the type of phage, as previously postulated. Romulus-containing powders exhibited a higher percentage of suitably sized particles, when compared to podovirus

LUZ19-containing particles. In contrast, the reduction in phage titre post spray drying (even in the presence of stabilising agents) was higher for Romulus phage (>2.5 log reduction), compared to LUZ19 (<1 log reduction), possibly as a consequence of shear stress on the long tail structure of the myovirus Romulus [57]. Therefore, the utilisation of dry powder phage therapy for treatment of pulmonary infection must rely on a compromise between particle size and phage survival, a fine balance which is evidently phage specific and very much dependent on production and storage conditions.

Stimuli Responsive Systems

Alongside the stability and release-location of bacteriophage, the release-kinetics from the delivery matrix must also be considered. Stimuli-responsive systems should remain kinetically silent, unless a 'burst release' of the therapeutic agent is initiated in response to an external stimulus (e.g. temperature, pH, light, ultrasound or biomarker signals). Thereupon, adequate dosage of the antimicrobial is rapidly delivered to the correct physiological location in response to a successful bacterial infection. By avoiding systematic dosage of the often delicate biological cargo, triggered phage release systems can in principle avoid reduction in viable phage count commonly associated with exposure to biological fluids/ temperature fluctuations [58]. Moreover, triggered release systems can help ensure bacterial pathogens are not exposed to sub lethal doses of phage, making the phage more effective and slowing evolution of bacterial resistance.

Triggered-release phage systems are being developed with the potential for treatment of wound infection. Such systems are designed as modifications of existing wound dressing

materials such as non-woven polymers, in order to incorporate stimuli-responsive phage delivery systems. One such system employs the thermally responsive polymer poly(N-isopropylacrylamide) (PNIPAM), which undergoes a reversible, temperature-dependent phase transition at the lower critical solution temperature (LCST), manifesting as an unambiguous change in polymer volume. Control of the LCST was established via formulation with allylamine (ALA), allowing the system to undergo a transitional collapse at 34 °C. Impregnation of PNIPAM-co-ALA nanospheres with *S. aureus* phage K, and subsequent grafting to a non-woven polypropylene 'dressing' was achieved via amine coupling to plasma deposited maleic anhydride. Utilising the proximity of the polymer's morphological change to healthy skin temperature (32 °C), thermally-triggered release of phage K from the nanospheres was engineered to occur in response to infected skin, which often displays an increase in skin temperature as large as 3.6 °C. Incubation of phage-loaded nanospheres with *S. aureus* ST228 showed release of the phage cargo and subsequent cell lysis at 37 °C, whilst the bacterial lawn remained confluent at temperatures associated with healthy skin [59].

In addition to secondary physical stimuli, primary biomarker signals have been utilised to trigger the release of phage K for the treatment of infected skin wounds. Hyaluronidase (HAase) is an important virulence factor known to be secreted by pathogens associated with skin infection, including *S. aureus*. Degradation of hyaluronic acid (HA) in the skin by HAase is thought to aid bacterial invasion of tissue via cleavage of the β -1,4 position in HA, hence allowing the enzyme to act as a spreading factor within the process of bacterial pathogenesis [60]. A HA/HAase system has been developed comprising of a dual-layered hydrogel matrix which allowed HAase to trigger release of phage K for treatment of pathogenic skin infection [61]. Photo-cross-linked HA methacrylate (HAMA) hydrogels were used to cap and seal a

lower reservoir layer of phage K containing agarose hydrogel. Clear visual signs of enzymatic degradation and subsequent phage release (ca. 10^5 pfu/ml) were observed when incubated with the supernatant of a wide range of *S.aureus* isolates. Both aforementioned examples of the incorporation a bacteriophage delivery system to a dressing platform exemplify the potential for future phage therapy, where phage virions are incorporated into a compatible carrier matrix.

Examples of photo-responsive systems utilizing bacteriophage, either within a 3 dimensional supramolecular hydrogel for cell culture and release [62], or in the derivation of a virus-like particle (VLP) for the photocaged delivery system of the anticancer drug doxorubicin [63], demonstrate the wide-reaching applications of stimuli-responsive phage technologies. In particular, release systems based on VLPs have made a promising start within biomedical research for both diagnostic and therapeutic roles [64]. As the recombinantly expressed and non-infectious structural analogues of virus particles, VLPs still possess native viral recognition elements. When appropriately functionalised, the clinical tunability of such particles allows them to act as candidates for a multitude of medical purposes, including drug delivery applications and tumour imaging.

Bacteriophage Endolysins

In addition to using whole phage as a potential alternative to conventional antibiotics, there has been recent interest in using phage derived products, specifically endolysins as therapeutics. Encoded by the phage genome, these small molecules are transcribed and synthesised within the bacterial host's cytoplasm following phage infection. They are then

translocated through the cytoplasmic membrane during the late stage of the infection cycle and are responsible for breaking down the bacterial cell wall, thus facilitating the release of the newly formed phage virions from the infected cell [65-67]. The primary benefit of using endolysins as opposed to whole phage lies with the elimination of genetic material from the therapeutic, thus eliminating the possibility of any genetic transfer events previously demonstrated with certain temperate phage [68]. Whilst bacteriophage undergoing consideration for medicinal use are incapable of such transduction, removal of phage genes entirely may help to subdue any concerns surrounding the opening of 'Pandora's Box' in terms of encouraging any phage assisted genetic mobilization. This may also help to speed regulatory acceptance of these new therapeutics. Furthermore, there are currently no identified resistance mechanisms towards phage lysins, indicative of the highly conserved, essential bacterial cell wall target sites of the phage encoded enzymes [69]. Unlike many antibiotics, lysins are specific in their target bacterial strain and have shown the ability to eliminate staphylococcal biofilms including persister cells (dormant, drug tolerant variants) [70-72]. The primary disadvantage of using lysins compared with whole phage is that they are not amplified in the host cell, thus larger doses are required for antimicrobial effect.

A number of different endolysins have been isolated, characterised, and produced recombinantly, demonstrating lytic activity against a range of bacterial species (including *Acinetobacter baumannii*, *Bacillus anthracis* and *Streptococcus pyogenes* [73-76]). Owing to the specific advantages over whole phage therapy, there is currently an endolysin based medical device registered for human use. Staphefekt™, marketed by Microcos consists of an endolysin active against *S.aureus* which can be used on intact skin for the treatment of conditions such as eczema, acne, rosacea and skin irritation. Whilst it is not licenced for use

in open wounds or within the medical setting at present, future clinical trials are expected to explore the possibility of developing Staphitekt™ into a clinical treatment [77]. Similarly ContraFect Corporation have developed an endolysin, CF-301, in collaboration with The Rockefeller University for the treatment of *S.aureus* associated bloodstream infections. As the first lysin to enter clinical trials in the US, the recently completed phase I trial results in healthy volunteers have shown promising results with no adverse clinical safety signals observed. CF-301 has shown potency in combination with approved anti-staphylococcal agents and in the eradication of methicillin resistant *S.aureus* biofilms [78]. Additionally CF-301 has been granted Fast Track Designation from the FDA in order to expedite its clinical assessment, a clear indication of how lysins have the potential to fill unmet medical needs.

Additional protein engineering approaches have also enabled lysins to be developed for Gram-negative pathogens which are usually considered to be recalcitrant to endolysin treatment [79]. Artilysin®s, consisting of an endolysin and an amphipathic or polycationic lipopolysaccharide-destabilizing peptide, are able to successfully penetrate the outer membrane of Gram-negative bacterial cells. Through disruption of ionic and hydrophobic forces within the protective outer membrane, these engineered fusion proteins are then able to degrade the peptidoglycan cell wall [80]. Artilysin®s have shown bactericidal efficacy both *in vitro* and *in vivo* against *P. aeruginosa* and *Acinetobacter baumannii* [81,82]. Similarly, ‘artilysation’ of endolysins effective against Gram-positive bacteria has shown to improve enzymatic and antibacterial activity compared to the wild type enzyme, through addition of a peptide selected to improve cell wall affinity [83].

However, as a relatively recent development, there are limited examples of successful delivery systems capable of carrying such antimicrobial cargo. One such study has successfully stabilized a staphylococcal endolysin, LysK (active against both methicillin and vancomycin resistant *S. aureus*), through complexation with polycationic polymers. Complexation resulted in increased activity, retention of specificity, and increased stability at physiological temperature. An increase in stability was also seen at 22°C, with poly-L-lysine and 10mM NaCl, manifesting as an increase in the half-inactivation time from 2 days for the free enzyme, to 2 months for complexed LysK [84]. It is believed that the change in kinetic properties of LysK upon complexation is likely a result of charge redistribution in the bacterial cell wall, following interaction between the cationic polyelectrolytes and the negatively charged surface proteins enclosed within the wall itself.

As previously stated, stimuli responsive materials are of particular interest as they allow for the controlled release of therapeutic cargo. The truncated form of LysK, denoted CHAP_K, consists of the single catalytic domain and has shown to exhibit lytic activity both *in vitro* and *in vivo* and against staphylococcal biofilms [72,85,86]. Exploiting synergistic effects with the bacteriocin lysostaphin, temperature responsive polymeric nanoparticles (PNIPAM) have demonstrated controlled release of the enzyme cocktail at an elevated temperature associated with infection *in vitro*. Formulated into a prototype wound dressing, nanoparticles containing the enzybiotics were anchored onto non-woven polypropylene via plasma activation of the surface and demonstrated statistically significant cell lysis at the elevated temperature (>4 log reduction in cell count), compared to the lower temperature associated with uninfected skin (<1 log reduction) [87].

Additional studies have investigated possible transportation methods for the delivery of endolysins to specific infections sites. The GI tract is capable of harbouring a range of pathogenic bacteria, many of which are difficult to treat (especially with a biopharmaceutical agent), owing to the harsh conditions found throughout the alimentary canal. Delivery of the endolysin CP25L, active against *Clostridium perfringens* (*C. perfringens*), to the GI tract has the potential to treat a range of diseases, including necrotic enteritis, gas gangrene and many common forms of food poisoning. Employing a dominant resident of the human intestinal flora, *Lactobacillus johnsonii* researchers have engineered this probiotic microbe to express CP25L, demonstrating successful production and secretion *in vitro* with retention of enzymatic lytic activity against *C. perfringens*. This engineered system provides a dual approach to targeting bacterial colonisation of the GI tract (initially via use of a probiotic microbe with the potential to be used as a competitive exclusion agent for the control of *C. perfringens* in its own right), and secondly through the secretion of an active endolysin with the potential for directed delivery to the gut [88,89].

Conclusion and Future Perspective

Current research into bacteriophage as an alternative or complementary treatment option for infectious diseases has shown encouraging results with a number of phage cocktails and products entering clinical trials. The development of phage therapy in Eastern Europe, in particular at the Eliavia Institute in Tbilisi, Georgia, has laid the foundation for the potential implementation of global phage treatment in the 21st century. Recent research into phage delivery systems has shown advantages over the administration of unprotected phage including enhanced bioretention, stability and protection from harsh environmental

conditions or inactivating agents. Optimisation of such carrier/support systems has the potential to enhance phage therapy to treat a range of infections.

However, despite the current success in the development of phage based therapeutics and the relatively large number of phage products in the pipeline, one of the major hurdles for future phage therapy will be clinical approval. The need to address the antibiotic vacuum is currently driving a call for a more economically viable drug approval process, specifically to allow phage to enter clinical trials whilst being relieved of the rigid and time consuming regulatory framework associated with classical clinical trials [19]. In recognition of the unmet medical requirement for the development of novel antimicrobials, a recent (2015) amendment to the Federal Food, Drug and Cosmetic Act was introduced in the US proposing a modified pathway for the approval of antibacterial drugs within a highly defined, limited population. Denoted the Promise for Antibiotics and Therapeutics for Health (PATH) Act [90], this modification to current legislation may allow for the approval of certain antibacterial agents for use within a defined population for treatment of a serious infection, whilst circumventing restrictive, conventional clinical trials. This type of adaptive licencing, alongside possible implementation of compassionate use guidelines such as the Right to Try Act of 2015 [91] (allowing unlicensed phase I experimental drugs, biological products or devices to be used by patients diagnosed with a terminal illness), are encouraging signs that some obstacles facing future phage therapy are being addressed from a regulatory standpoint. The current clinical framework for conventional antibiotic approval may indeed prove unsuitable for whole phage which, as self-replicating biological entities are unlikely to conform to classical drug analysis. Following successful bacterial binding and infection, there is likely to be a significant increase in local phage concentration which may result in an

underestimation of phage efficacy via standard PK/PD analysis. Conversely, phage endolysins which show a far greater similarity to chemical antibiotics may conform more successfully to current clinical assessment.

The future of bacteriophage therapy using targeted delivery systems looks promising for the treatment of serious, chronic and multidrug resistant bacterial infections, owing to the capability of controlling the pharmacokinetics/ dynamics of the phage, alongside offering protective and stabilising effects. However in order to continue driving the development of phage based therapeutics and the possible adaptation of regulatory pathways towards their approval, focus must be placed on stringent pre-clinical evaluation, with particular emphasis placed on phage concentration (MOI), biodistribution and suitable *in vivo* modelling. The current antibiotic crisis facing modern medicine appears to be shifting the paradigm in favour of non-traditional therapy, affecting both development and approval strategies. Thus the next 5-10 years will be an intriguing time for bacteriophage research, offering the potential to see application within the clinical setting.

Executive Summary

Encapsulation

- Liposomal encapsulation can provide effective protection of phage from degradative effects including neutralisation by anti-phage antibodies and systematic clearance *in vivo*. Liposomes provide enhanced cellular uptake of phage in order to target intracellular diseases such as pneumonia and tuberculosis.
- Nanoemulsions can stabilise phage via aqueous encapsulation manifesting as an increase in shelf life and higher infectivity rates.

- Encapsulation within modified alginate microspheres provides a protective strategy capable of preventing phage inactivation under simulated gastric conditions. Addition of stabilising agents such as maltose and maltodextrin allow for the formulation of dry powders containing phage with retention of phage activity and stability.

Immobilisation

- Anchoring to surfaces via plasma activation has successfully immobilised active phage capable of causing bacterial lysis. Retention of activity was achieved for several months under the correct conditions.

Polymeric Formulations

- Complexation with naturally occurring polymers can provide protection against temperature, pH and UV, increasing both the versatility and long term stability of complexed phage. Increased activity and stability has also been seen with phage endolysins through complexation with polycationic polymers.

Aerosol Formulations

- Aerosol formulations for treatment of pulmonary infection can effectively incorporate phage, both in liquid form for use in nebulizers and in dry form for use in inhalers.
- By careful control of particle size, phage containing aerosols can successfully target pulmonary infection. However delivery formulation, storage conditions and the use of stabilisers must be considered individually for each phage administered in order to retain viability and stability.

Stimuli Responsive Systems

- Controlled release of both phage and phage endolysins offers benefits in terms of preventing unnecessary administration of an antimicrobial and protection of the cargo from environmental conditions until it is required.

- Successful triggered release systems have been shown to release phage/ phage lysins as a response to various stimuli including temperature and bacterial toxin production for the treatment of wound infection.

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